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Research Note

Comparison of Characteristics of the *nodX* Genes from Various *Rhizobium leguminosarum* Strains

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We have analyzed the nucleotide sequences of the *nodX* genes from two strains of *Rhizobium leguminosarum* bv. *viciae* able to nodulate Afghan peas (strains A1 and Himalaya) and from two strains of *R. leguminosarum* bv. *trifolii* (ANU843 and CSF). The *nodX* genes of strains A1 and ANU843 were shown to be functional for the induction of nodules on Afghan peas. To analyze the cause of phenotypic differences of strain A1 and strain TOM we have studied the composition of the lipochitin-oligosaccharides (LCOs) produced by strain A1 after induction by the flavonoid naringenin or various pea root exudates. The structural analysis of the LCOs by mass spectrometry revealed that strain A1 synthesizes a family of at least 23 different LCOs. The use of exudates instead of naringenin resulted only in quantitative differences in the ratios of various LCOs produced.

Most wild-type pea cultivars from Afghanistan are resistant to nodulation by European and North American *Rhizobium* strains due to the presence of the single genetic locus *sym2*^{A} (Kozik et al. 1995; Lie 1978). *R. leguminosarum* bv. *viciae* strains able to overcome this resistance were initially isolated from soils in the Middle East (Winarno and Lie 1979) and later from other regions of the world (Ma and Iyer 1990). It was shown by Southern hybridization (Ma and Iyer 1990) that all these strains carry an additional gene, designated *nodX*, that was first described in strain TOM (Davis et al. 1988). By functional complementation the presence of a *nodX* gene was also predicted in *R. leguminosarum* bv. *trifolii* strains but its function in this biovar was unknown (Lewis-Henderson and Djordjevic 1991b; Canter Cremers et al. 1988). There is evidence that the *nodX* gene from the *R. leguminosarum* bv. *viciae* strain TOM encodes an *O*-acetyl transferase that transfers an acetyl group to the reducing terminal residue of lipochitin-oligosaccharides (LCOs) produced by rhizobia (Firmin et al. 1993). The LCOs, also called Nod factors, are the bacterial signal molecules produced in response to secretion of flavonoids by the host plants. These flavonoids activate the transcription of the bacterial genes (called *nod*, *nol*, or *noe* genes) that are involved in the synthesis of the Nod factors. The modifications present on the LCO core structure are important determinants of the host specificity in rhizobia-legume symbioses (for reviews see Bladergroen and Spaink 1998; Dénarié et al. 1996). The finding that the rhizobial *nodX* gene controls the O-acetylation of the reducing terminal residue of LCOs suggests that *sym2*^{A}-carrying peas require rhizobial strains producing doubly acetylated Nod factors for nodulation. Although a large collection of *R. leguminosarum* strains nodulating Afghan peas has been described (Ma and Iyer 1990), all the data on the genetic and biochemical control of the host specificity of symbiosis with *sym2*^{A}-carrying peas strains were obtained only from the study of the *nodX* gene from *R. leguminosarum* bv. *viciae* TOM (Winarno and Lie 1979).

The aim of this work was to compare the characteristics of the *nodX* genes present in different wild-type *R. leguminosarum* strains with the homologous *nodX* gene from *R. leguminosarum* bv. *viciae* TOM, whose sequence and function have been described (Firmin et al. 1993; Davis et al. 1988). We have analyzed two other *R. leguminosarum* bv. *viciae* strains, A1 (Chetkova and Tikhonovich 1986) and Himalaya (Lie 1981), and two *R. leguminosarum* bv. *trifolii* strains, ANU843 (Rolfe et al. 1985) and CSF. Strain A1 was isolated from soils of northwestern Russia based on its ability to elicit nodules on Afghan peas and its high competitive ability in co-inoculation experiments. Nodulation by strain A1, in contrast to that by strain TOM, is not inhibited by various *R. legumi-*

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Nucleotide and/or amino acid sequence date have been deposited in the GenBank data base as accession numbers AF031146, AF084223, and AF084224.

nosarum strains that do not contain the *nodX* gene, a phenomenon called "competitive nodulation blocking" (Dowling et al. 1987; Chetkova and Tikhonovich 1986).

The *nodX* genes from strains A1, Himalaya, and CSF were cloned following PCR (polymerase chain reaction) amplification of bacterial genomic DNA with specific primers for the *nodX* gene based on the *nodX* sequence from *R. leguminosarum* bv. *viciae* TOM described earlier (Davis et al. 1988). The sequence from the strain ANU843 *nodX* gene was determined by sequencing 1,946 nucleotides (nt) downstream of a point within the *nodN* gene located on plasmid pJJ258 (Table 1). The data show that the *nodX* gene is positioned 284 nt downstream of *nodN* (Lewis-Henderson and Djordjevic 1991b). The nucleotide sequences of the cloned fragments were determined completely in the case of the *nodX* genes from strains A1 and ANU843, or partially in the case of strains Himalaya and CSF. The ANU843 and CSF sequences have been deposited in the GenBank data base (accession numbers AF031146, AF084223 and AF084224). The *nodX* genes appear to be very similar to each other and to the known *nodX* sequence of strain TOM. The closest similarity was observed within *R. leguminosarum* bv. *viciae* since the complete coding sequence of the *nodX* gene from strain A1 differed from that of strain TOM in only 3 nucleotide residues (listed in the legend to Figure 1). This result was confirmed by sequencing a clone derived from independently isolated chromosomal DNA. Surprisingly, the sequenced part of the *nodX* gene from strain Himalaya (600 out of 1,101 bp) did not display any differences with the homologous part of the *nodX* gene from strain A1. The *nodX* genes from the *R. leguminosarum* bv. *trifolii* strain ANU843 were 94% homologous to those of strain TOM (71 bp different in the coding region of 1,104 bp). As shown in Figure 1, the degree of similarity was somewhat higher at the level of the predicted amino acid sequence, where only 23 amino acids are different between the predicted NodX proteins of strains ANU843 and TOM. Interestingly, the sequence of the *nodX* homologue of strain CSF shows a replacement of leucine 41 by a stop codon and also a frame shift in the middle of the gene (Fig. 1), indicating that this *nodX* gene is not functional and is maintained in the genome as a degenerate copy.

To test the function of the cloned *nodX* genes from *R. leguminosarum* bv. *trifolii* ANU843 and from *R. leguminosarum* bv. *viciae* A1, we tested their capacity to confer on *R. leguminosarum* bv. *viciae* 248 the ability to nodulate Afghan peas. For expression of the cloned *nodX* genes in rhizobia, we have re-cloned them under the control of the *nodABCIJ* promoter into a broad-host-range plasmid. The resulting *nodX*-carrying plasmids pMP3219 and pJJ392 (containing *nodX* from strains A1 and ANU843, respectively) were introduced into strain 248, which lacks the *nodX* gene, and the resulting transconjugant strains were used for inoculation of wild-type Afghan pea line L2150. For inoculation of line L2150, use was made of the gravel test system as described previously (Ovtsyna et al. 1998). For each experiment 12 plants were analyzed and nodulation was scored after 6 weeks.

The results showed that both *nodX* genes confer the ability to nodulate Afghan pea as efficiently as the positive control

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains[a]		
248	*Rhizobium leguminosarum* bv. *viciae* wild type	Josey et al. 1979
A1	*R. leguminosarum* bv. *viciae* wild-type strain nodulating on Afghan peas, isolated from soils of northwestern Russia	Chetkova and Tikhonovich 1986
TOM	*R. leguminosarum* bv. *viciae* wild-type strain nodulating on Afghan peas, isolated in Turkey	Lie 1978
ANU843	*R. leguminosarum* bv. *trifolii* wild-type strain	Rolfe et al. 1985
Himalaya	*R. leguminosarum* bv. *viciae* wild-type strain nodulating on Afghan peas, isolated from a nodule of *Vicia faba* growing in the Hindukush region	Lie 1981
CSF	*R. leguminosarum* bv. *trifolii* wild-type strain isolated from nodules of *Trifolium pratense*	This work
Plasmids		
pRL1JI	Sym plasmid of the *R. leguminosarum* bv. *viciae* strain 248	Johnston et al. 1978
pRK2013	IncColE1, Tra^+, Km^r	Ditta et al. 1980
pET3d	IncColE1, expression vector based on the bacteriophage T7 promoter, Amp^r	Studier et al. 1990
pIC20R	IncColE1, cloning vector, Amp^r	Marsh et al. 1984
pGEM-5Z(+)	IncColE1, cloning vector, Amp^r	Promega, Madison, WI
mp8	Bacteriophage M13-derived cloning vector	Messing and Vieira 1982
pMP3510	IncP, multiply cloning vector containing *nodABCIJ* promoter, Tc^r	Spaink et al. 1995b
pWB5a	IncP, multicopy cloning vector, Tc^r	Hirsch et al. 1985
pMP3218	Plasmid pET3d containing the *nodX* gene of strain A1 under control of the T7 promoter, Amp^r	This work
pMP3219	Plasmid pMP3510 containing the *nodX* gene of strain A1 under control of the *NodABCIJ* promoter, Tc^r, Amp^r	This work
pJJ258	Plasmid pBluescript SK(+) carrying a 3-kb *Bgl*II-*Bam*HI fragment (present in pRT290) containing a partial *nodM* gene, and the *nodN* and *nodX* genes from strain ANU843	This work and Djordjevic et al. 1986
pJJ390	Plasmid pWB5a carrying the strain ANU843 *NodABCIJ* promoter as a 242-bp fragment[b] in the *Bam*HI site of the polylinker, Inc-P, Tc^r	This work
pJJ392	Plasmid pJJ390 carrying the strain ANU843 *nodX* gene as a *Pst*I-*Xba*I fragment from pJJ258, Tc^r	This work

[a] Rhizobial strains were gron in B^- medium supplemented with phosphate buffer (100 mg/ml; van Brussel et al. 1977).

[b] This fragment was first isolated as a 242-bp *Bam*HI-*Bgl*II cartridge by amplification with the proof-reading Deep Vent DNA polymerase (New England Biolabs, Hertfordshire, U.K.) and the oligonucleotide primers 5′-CCAGGATCCATGCAAAGCTCCAA-3′ (located in complementary sequence of *nodA*) and 5′-CTTAGATCTCATTCTCGATCCAC-3′ (located in sequence of *nodD*).

strain TOM. As judged by the dry weight of the plants after 6 weeks of growth, the nodules apparently also fixed nitrogen efficiently. Under the test conditions used, strain 248 was completely unable to induce nodules on Afghan pea plants. In conclusion, the introduction of the separate *nodX* gene from strains ANU843 and A1 was sufficient to confer on strain 248 the ability to elicit effective nodules on Afghan peas.

We have studied whether the presence of the *nodX* gene in *R. leguminosarum* bv. *viciae* A1 correlates with the production of doubly acetylated LCOs, as was shown for strain TOM (Firmin et al. 1993). First, the ability of strain A1 to produce Nod factors was studied by means of thin layer chromatography (TLC). Cells of strain A1 were grown with [1-^{14}C]-D-glucosamine in the presence of the appropriate inducer. The culture fluids were extracted into *n*-butanol, concentrated, and applied to reverse-phase TLC plates by published methods (Spaink et al. 1994b). The results show that, with several commercial inducers and root exudates from different pea lines (lines L32, L1887, and L6559), strain A1 produces LCOs in much larger quantities than does strain TOM, and

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          1
A1        MGPSNEHSSG HRNNFDLLRL FAACQVMFSH AWNWLHLGDP LNGTWVFDLL
ANU843    MGPSNEHSSG HRNNFDLLRL FAACQVMFSH AWNWLHLGDS LNGTAAFNLL

          51
A1        FSAPGVAIFF LISGFLVTDS YIRSSSAASF FVKRSLRIFP ALFVNIAVME
ANU843    FSTPGVAIFF VISGFLVTDS YIRSSSEASF FVKRSLRIFP ALFVNIAMME

           101
A1        LALLVTGGLN VTGILQYLFY FTVYILTAAR IWAVYFTYEP YTMSGFYGAS
ANU843    LALLVTGGLN VTGILQYLCY FTVYMLTAAR IWAVYFTYEP YTMSGFYGVS

          151
A1        DPSGVLWTLT VELTFYLTLP MLLEIWRRWK RAGALVVAVA ALGSWVMAQH
ANU843    DPSGVLWTLT VELTFYLTLP TLLEIWRRWK RAGALVVAVA ALGSWVMAQY

          201
A1        FNITDKYNPC LSVTAGPTFW IFSMGVLARL YWHRVSKIFE GKLLWWLATH
ANU843    FNITDRYNPF LSVTVGPTFW IFSMGVLARL YWHRVSRIFE GKLLWWLAAH

          251
A1        LAITWWVAGT SAAFISINNA APVDAFRIAV LAGLVLSAAH SLPRPNLLRR
ANU843    LAITWWVAGT SAAFISINNA APVDAFRIAI LAGLVLSAAY SFPRPNLLRG

          301
A1        QDLSYGIYLY HMLVMHTLIA IGWVGHWWLW IVEPVGTVAL AALSWALIEQ
ANU843    QDLSYGIYLY HMLVMHTLIG MGWVGHWWLW IVEPVGTVAL AALSWALIEK

          351              368
A1        PAMKLRTSLV ARRLSVA
ANU843    PAMKLRTSLV ARRLSVA
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Fig. 1. Comparison of the protein sequences encoded by the *nodX* genes from the *Rhizobium leguminosarum* bv. *viciae* strain A1 and the *R. leguminosarum* bv. *trifolii* strain ANU843. Differences between the NodX sequence from strain A1 and the previously published sequence from strain TOM (Davis et al. 1988) are boxed, or underlined when the difference does not lead to a difference in the amino acid encoded. Differences found with the GenBank submission for *nodX* of strain TOM (X07990) are as follows: T conversion to C (position 353); T conversion to C (position 460); T conversion to G (position 960). The boxed leucine in the predicted NodX sequence of strain ANU843 indicates the position where a stop codon occurs in the sequence obtained from strain CSF, whereas an arrow indicates the position of a frame-shift mutation. The *nodX* genes from strains A1, Himalaya, and CSF were cloned by PCR (polymerase chain reaction). Specific primers for the *nodX* gene were designed according to the DNA sequence of the *nodJ-nodX*-containing region of strain TOM (Davis et al. 1988): upstream primer, oMP199 (5′-CCATGGGACCATCCAATGAAC-3′) and downstream primer, oMP196 (5′-TTAAGCGACGGAAAGCCTTC-3′) (Isogen Bioscience, Maarssen, The Netherlands). The DNA sequence amplified with these primers covered the structural part of the *nodX* gene, 1.1 kb in size. Amplification was performed with the high-fidelity enzyme *Pwo* DNA Polymerase (Boehringer, Mannheim, Germany) for 25 cycles in a RoboCycler PCR machine (Stratagene, La Jolla, CA). The amplified 1.1-kb DNA fragment was then purified and amplified for an additional 7 cycles with AmpliTaq Polymerase (Sigma Chemical, St. Louis, MO) to facilitate cloning of the PCR product into a pGEM T-vector (with linearized pGEM-5Z(+) vector with single-strand thymidines on the 3′ ends obtained from Promega, Madison, WI). Cloned genes were sequenced in the T-vector by the dideoxy chain termination method (Sanger et al. 1977). To determine the whole sequence of the *nodX* gene, *AccI* and *NarI* fragments of this gene were subcloned into M13mp8 and pIC20^{r} cloning vectors, respectively. Plasmid containing *nodX* fragments with deletions resulting from digestion of the T-vector-*nodX* plasmid with *Acc*I, *Apa*I, and *Nar*I endonucleases followed by re-circularization of the plasmid were also used for sequencing.

comparable with those of standard *R. leguminosarum* bv. *viciae* 248, as judged by quantitation of the radioactive spots corresponding to LCOs (data not shown). Of the commercial inducers, the best was naringenin at a concentration of 3 µM, but the best LCO production was achieved when root exudates from European pea line L32 were used for induction.

Based on these results, LCOs produced by *R. leguminosarum* bv. *viciae* A1 were obtained from 2-liter cultures grown in the presence of the inducers naringenin or pea root exudate. Root exudates from European pea line L32 and from Afghan pea line L6559 were chosen as the best inducers on the basis of the labeling experiments. As a control, LCOs were purified from 2-liter cultures of *R. leguminosarum* bv. *viciae* 248 induced by naringenin. LCOs were extracted into *n*-butanol and purified by reverse-phase HPLC (high-pressure liquid chromatography) as described previously (Spaink et al. 1995a). The HPLC profiles of LCOs induced by naringenin in strain A1 and in strain 248 are shown in Figure 2. The HPLC profiles of LCOs derived from these strains are very similar except that at least one additional peak was present in the HPLC profile of strain A1, eluting with a retention time of 35 min (Fig. 2B). Diode array analysis showed that this peak has a specific absorbance maximum at 304 nm (data not shown). HPLC profiles of LCOs induced by root exudates of European and Afghan pea lines in strain A1 were indistinguishable except for a quantitative difference in the relative intensity of the peak at 34 min (Fig. 2C and D). More obvious differences were observed with the naringenin-induced culture in the relative intensities of the different peaks. The additional peak observed in the LCO pattern from strain A1, compared with that from strain 248, migrates at a position similar to that for the doubly acetylated, pentasaccharide Nod factors characteristic of *R. leguminosarum* bv. *viciae* TOM (Firmin et al. 1993). For analysis of the LCO molecular structures, separate fractions were collected after HPLC (one sample per minute).

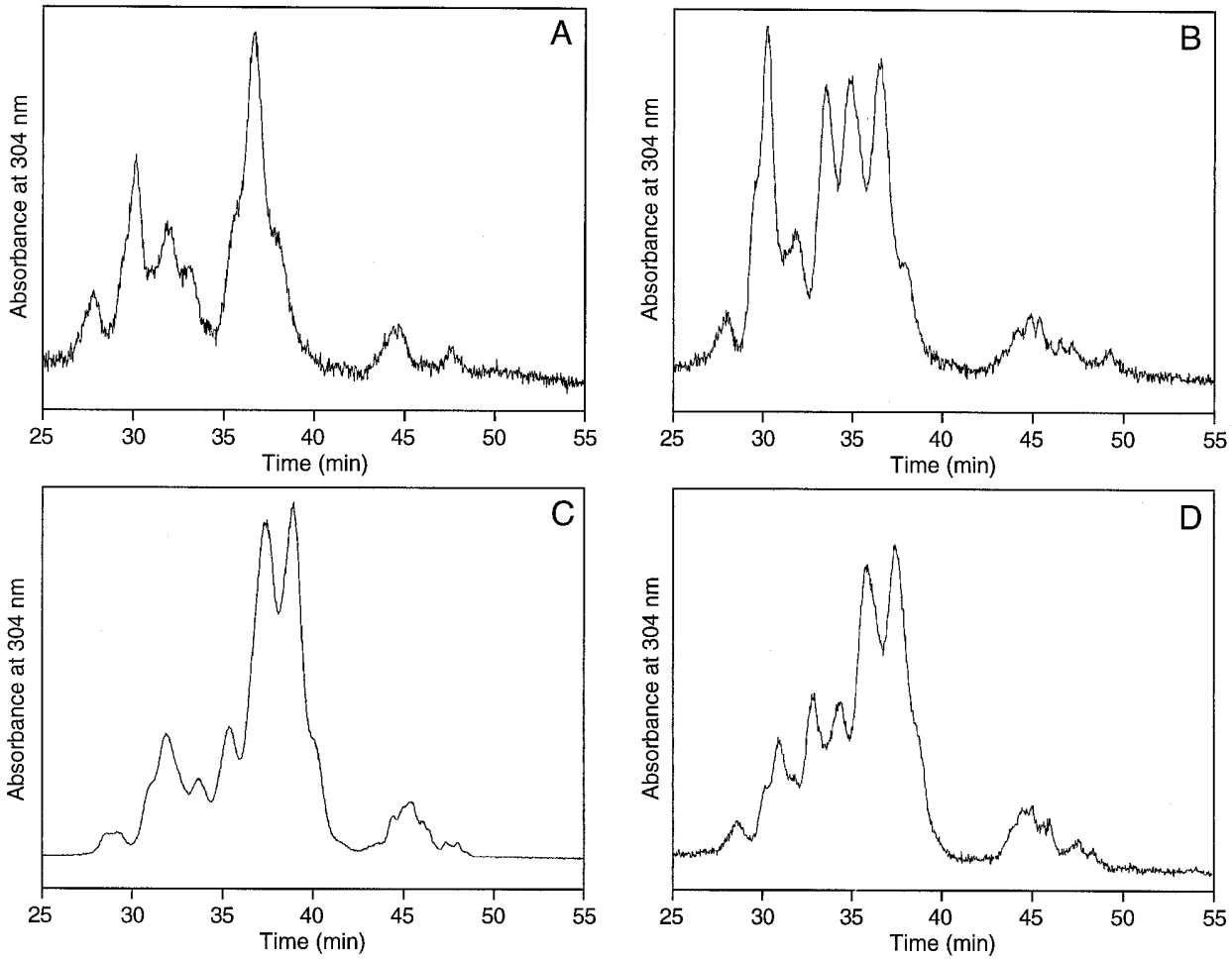

Fig. 2. HPLC (high-pressure liquid chromatography) profiles of Nod metabolites produced by the *Rhizobium leguminosarum* bv. *viciae* strains 248 and A1 induced by naringenin or pea root exudates. **A,** Strain 248 induced with naringenin. **B,** Strain A1 induced with naringenin. **C,** Strain A1 induced with European pea exudate. **D,** Strain A1 induced with Afghan pea (line L6559) exudate. For the preparation of exudates, pea seeds (50 per each line) were surface sterilized with concentrated sulfuric acid, washed thoroughly with sterile water, and germinated on minimal agar plates for 3 days. Then, seeds were transferred to filter paper in petri dishes with water for the secretion of the exudates. After 3 days the exudates were collected, seeds and filter paper were washed with water, and these solutions were pooled. The exudates were purified by filtering through Whatman no.1 filter paper, evaporated under vacuum, and dissolved in 3 ml of 30% ethanol (1/10 of the initial volume) and used in a 1:500 dilution. Separation of lipochitin-oligosaccharides (LCOs) was carried out on a reverse-phase column (5 mm, 4 × 250 mm; Pharmacia LKB, Uppsala, Sweden) with a gradient running from 30/70 acetonitril/water to pure acetonitril at a flow rate of 0.7 ml/min.

The structures of the Nod factors produced by strain A1 were determined by fast atom bombardment (FAB) mass spectrometry and collision-induced dissociation (CID) tandem mass spectrometry. It was shown that strain A1 produces a large variety of LCOs (Table 2). As expected, the additional peak present in the LCO profile from strain A1 (at 35 min retention time) correlates with the presence of doubly acetylated, pentasaccharidic LCOs. In addition to these Nod factors, several species were found to be produced by A1 that were not reported from the previously analyzed *R. leguminosarum* bv. *viciae* strains, namely LCOs with C18:2 and C18:3 fatty acids (Table 2; Spaink et al. 1994a). Qualitative variations in the LCOs produced when different inducers were used were not detectable. However, considering the mass spectrometric data alongside the HPLC chromatograms (for simplicity only shown at 304 nm absorbance in Figure 2), it is evident that there are two quantitative differences between the LCOs induced by naringenin and those induced by root exudates: (i) naringenin-induced bacteria produce relatively very little of the trisaccharidic species, whereas the bacteria induced by root exudates produce more abundant trisaccharidic LCOs; and (ii) in naringenin-induced bacteria more pentasaccharidic LCOs were detected than in exudate-induced fractions, where tetrasaccharides were the major products detected. In summary, pea root exudates induce abundant synthesis of short (3 to 4 monosaccharide residue) LCOs, while in naringenin-induced fractions the LCOs with longer backbones were observed in relatively large amounts.

To conclude, strain A1 produces a wide spectrum of Nod factors, including the doubly acetylated species reported previously from strain TOM (Firmin et al. 1993), and additional LCO species that are not commonly observed from the *R. leguminosarum* bv. *viciae* strains analyzed previously (Spaink et al. 1995b). It can also be noted, that under optimal conditions of induction, strain A1 was able to produce large total amounts of LCOs, typically of the order of 1 to 10 mg per liter. In contrast, it was reported that strain TOM produces only minor quantities of LCOs (Firmin et al. 1993), a result that was confirmed by some preliminary experiments in our laboratory (data not shown).

The presence of the *nodX* gene was also predicted for some *R. leguminosarum* bv. *trifolii* strains (Lewis-Henderson and Djordjevic 1991a; Ma and Iyer 1990). However, the presumed location of the *nodX* gene in *R. leguminosarum* bv. *trifolii* was shown to be different from that in *R. leguminosarum* bv. *viciae*, since in *R. leguminosarum* bv. *trifolii* hybridization was observed downstream of the *nodN* gene. The functional ability of the *nodX* gene from strain ANU843 was tested by intro-

Table 2. Lipochitin-oligosaccharides (LOCs) produced by the *Rhizobium leguminosarum* bv. *viciae* strain A1 in the presence of different inducers[a]

			Retention time (min)		
	Mass spectrometric data *m/z*[b]		Induction gene-inducing factor		
LCO structure	**M+H$^+$**	**MS-MS fragment ions**	**Naringenin**	**Afghan pea**	**European pea**
III (18:1, Ac)	892	468, 671 (EP, AP)	45–46	39–46.5	43–49
III (18:2, Ac)	890		45	39–44.5	43–46
IV (16:0, Ac)	1069		37–40	38–42	36–43
IV (16:1, Ac)	1067		33–34	33–38	33–37
IV (18:0, Ac)	1097		NC	NC	51–52
IV (18:1)	1053		37–42	38–46.5	40–43
IV (18:1, Ac)	1095		42–46	43–46.5	43–51
IV (18:1–OH, Ac)	1111	484, 687, 890 (AP)	32–35	34–38	35–38
IV (18:2, Ac)	1093		34–40 + 42–43	30–35 + 43	30–33, 35–36 + 40–44
IV (18:3, Ac)	1091		31 + 33–40	30–35 + 39–43	30–36 + 40–43
IV (18:4, Ac)	1089		32–33 + 35–38	33–39	33–40
V (16:0, Ac)	1272		31–37	32–39	31–41
V (16:0, Ac2)	1314	442, 645, 848, 1051 (AP)	37–38	36–43	38–43
V (18:0, Ac2)	1342	470, 673, 876, 1079 (EP)	NC	NC	50–52
V (18:1)	1256		31–34	33–35	33–36
V (18:1, Ac)	1298		37–46	36–46.5	38–49
V (18:1–OH, Ac)	1314		29–32	30–32	30–32
V (18:1, Ac2)	1340	468, 671, 874, 1077 (EP)	44–45	43–46.5	45–50
V (18:2, Ac)	1296		34–35	34–38	34–36
V (18:3, Ac)	1294		32–34	33–35	34–36
V (18:3, Ac2)	1336	Insufficient for MS–MS	38	38–42	38–41
V (18:4, Ac)	1292	462, 665, 868, 1071 (EP)	29–33	30–33	29–38
V (18:4, Ac2)	1334	462, 665, 868, 1071 (EP, AP)	33–35	32–38	33–38

[a] Abbreviations: EP = data obtained only from European pea exudate; AP = data obtained only from Afghan pea exudate; NC = not collected; MS = mass spectrometry.

[b] Positive-ion mode FAB (fast atom bombardment) mass spectra were obtained with MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at 10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mA and xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system running JEOL Complement software. CID (collision-induced dissociation) mass spectra were recorded with the same machine, with nitrogen as the collision gas in the third FFR collision cell, at a pressure sufficient to reduce the parent ion to one third of its original intensity. Monothioglycerol was used as the matrix, unless noted otherwise, when *meta*-nitrobenzylalcohol (*m*-NBA) was used. The HPLC (high-pressure liquid chromatography) fractions were dried and redissolved in 10 µl of dimethyl sulphoxide prior to mass spectrometric analysis. From each of the fractions a 1- to 3-µl aliquot was loaded into the matrix. The matrix used was thioglycerol for most FAB-MS spectra and all CID-MSMS spectra. In some cases *m*NBA containing a large amount of sodium was used for the FAB mass spectra. When the spectra run in *m*NBA were compared with those run in thioglycerol the relevant LCO signals were shifted 22 amu, corresponding to the formation of $[M+Na]^+$ rather than $[M+H]^+$ pseudomolecular ions, thus facilitating the correct assignment of the structures. When *m*NBA was used as the matrix, the sensitivity of the analysis was significantly reduced.

duction of the cloned gene into *R. leguminosarum* bv. *viciae* strain 248, which is not able to nodulate Afghan peas. The results show that the introduction of the separate *nodX* gene from strain ANU843 was sufficient to confer on strain 248 the ability to elicit effective nodules on Afghan peas. In agreement with these results is the finding that some classes of LCOs produced by the wild-type strain ANU843 do contain a second *O*-acetyl group attached to C6 of the reducing terminal saccharide (Philip-Hollingsworth et al. 1995; M. Olsthoorn, J. E. Thomas-Oates, and H. P. Spaink, *in preparation*).

Our data on the structures of the Nod factors of strain A1 are in complete agreement with the data of Firmin et al. (Firmin et al. 1993) that indicate that the O-acetylation directed by the NodX protein is confined to LCO molecules of pentasaccharidic chain length. Phenotypic differences between these strains, such as the insensitivity of strain A1 to "competitive nodulation blocking" can therefore not be assigned to the presence of the second acetyl group in the LCOs. The difference in susceptibility to blocking by other strains could be explained in two obvious ways. First, the difference could be due to differences in the production of NodX-independent minor LCO species produced by strain A1. Considering the technical impossibility of purifying these minor LCOs species this hypothesis would be very difficult to test. Second, the difference could be due to the seemingly large difference in the quantities of LCOs produced by strains TOM and A1 under laboratory conditions. We have shown that strain A1 also produces relatively high levels of LCOs in the presence of pea exudates, indicating a possible biological role for the production of higher levels of Nod factors. Therefore, future studies on the differences between strains A1 and TOM in symbiosis and competitiveness could be directed at determining whether there are differences in the regulation of the level of Nod factor production under laboratory and field conditions.

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